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The V Antigen of *Pseudomonas aeruginosa* Is Required for Assembly of the Functional PopB/PopD Translocation Pore in Host Cell Membranes

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Pseudomonas aeruginosa **efficiently intoxicates eukaryotic cells through the activity of the type III secretion-translocation system (TTSS). Gene deletions within the translocation operon *pcrGVH-popBD* abolish pore-forming activity of *P. aeruginosa* strains with macrophages and TTSS-dependent hemolysis. Here we investigated the requirements for PcrV, PopB, and PopD in pore formation by analyzing specific mutants using red blood cells (RBCs) and fibroblasts expressing green fluorescent protein fused to actin. Simultaneous secretion of three proteins, PopB, PopD, and PcrV, was required to achieve wild-type hemolysis and effector translocation. Deletion of *pcrV* in a cytotoxic strain did not affect secretion of PopB and PopD but abolished hemolytic activity and translocation of effectors into fibroblasts. Notably, the PcrV-deficient mutant was not capable of inserting PopD into host cell membranes, whereas PopB and PopD, but not PcrV, were readily found within membranes of wild-type-infected RBCs. Immunoprecipitation experiments performed by using a liposome model of pore assembly revealed a direct interaction between PopD and PopB but not between PopD and PcrV. Consequently, PcrV is necessary for the functional assembly of the PopB/D translocon complex but does not interact directly with pore-forming Pop proteins.**

Pseudomonas aeruginosa is a ubiquitous gram-negative opportunistic pathogen which frequently causes nosocomial infections. Patients with severe immunosuppression or underlying disease, such as cystic fibrosis and severe burn patients, are especially at risk of colonization and infection by *P. aeruginosa*. In a cystic fibrosis patient's lung, infection and the associated inflammatory reactions due to this bacterium are considered to be a predictor of morbidity and mortality. Once the infection has been established, eradication is difficult despite prolonged antibiotic therapy (36, 57).

P. aeruginosa, like other gram-negative plant and animal pathogens, utilizes a type III secretion-translocation system (TTSS) to intoxicate eukaryotic cells. This system allows the extracellularly located bacterium to inject its toxic products (effectors) directly into the host cell cytoplasm (13, 24). The secretion and translocation steps involve more than 20 proteins assembled into a needle-like structure called an injectisome (5, 12). Four exoenzymes (ExoS, ExoT, ExoY, and ExoU), which are secreted and translocated through the *P. aeruginosa* injectisome, paralyze normal cellular functions (20, 29, 60), thus enabling successful establishment of infection. All clinical isolates of *P. aeruginosa* possess the TTSS locus encompassing five operons coregulated by the central transcriptional activator ExsA (15, 20). Recent studies have shown that operons of the locus are expressed in 25 to 80% of strains, depending on the site of infection from which the strain collection comes (3, 15, 18, 28, 43). The phenotypes associated with TTSS-secreted proteins on cultured cells depend both on the eukaryotic cell type and on the combination of secreted effectors (11, 19, 30, 55).

Components involved in effector translocation across the host plasma membrane are encoded in the *pcrGVH-popBD* operon (Fig. 1A). PopB is a 391-amino-acid protein with two predicted α-helical transmembrane domains (amino acids 171 to 191 and 235 to 259) and two coiled-coil domains (amino acids 107 to 174 and 331 to 375). PopD is a 296-amino-acid protein possessing one predicted central transmembrane domain (amino acids 116 to 137) and, in addition, a C-terminal domain with the potential to form an amphipathic α-helix (amino acids 267 to 281) (Fig. 1B) (9, 47). When provided in *trans*, encoded by the *pcrGVH-popBD*-containing fragment, these products are able to complement *Yersinia pseudotuberculosis yopB* or *yopD* mutants deficient in delivery of the YopE effector to the host cell (23). When the homologous proteins of *Yersinia* spp., YopB and YopD, are expressed by *Yersinia*, they are able to associate with liposomes (53). Moreover, recent work has shown that in vitro oligomerization of recombinant PopB and PopD promotes binding to and disruption of artificial membranes by formation of ringlike structures (47). It has been proposed that those proteins participate in the formation of a translocation pore which allows active passage of toxins across the eukaryotic cell membrane (26, 41, 47).

PcrH is an 18.5-kDa acidic protein that binds to PopB and PopD (1, 47) and acts, in vivo, as a customized chaperone necessary for stabilization of Pop proteins (7). A third secreted protein of the operon is PcrV, also called the V antigen. Antibodies directed against PcrV protect cultured cells and model animals against *P. aeruginosa* infection, thus making PcrV a

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference(s)
Strains		
E. coli DH5α		Invitrogen
P. aeruginosa		
CHA	Mucoid, cytotoxic cystic fibrosis isolate	14, 54
CHAΔBD	CHA with the gentamicin cassette inserted within *popB* and *popD*	This study
CHAΔV	CHA with an internal deletion of the *pcrV* gene	This study
Plasmids		
pIA60	7-kb EcoRI fragment from CHA with *pcrGVH-popBD* and *exsCBA* in pUC18	This study
pIA101*	Ap^r-pUCP20-*gfp*mut3 with deletion of the *plac* −10 region	15
pUCGm	Ap^r, Gm^r	48
pPCR-Script	Ap^r, cloning vector	Stratagene
pUC18	Ap^r, cloning vector	New England Biolabs
pEX100T	Ap^r, cloning vector	49
pRK2013	Km^r, ColE1 mob^+ tra^+ (RK2) helper plasmid	34
pIApG	Fusion between the *pcrGVH-popBD* promotor (*pG*) and *gfp*mut3	This study
pJG1	Ap^r, 426-bp EcoRI/BamHI PCR fragment of *popB* in pUC18	This study
pJG2	Ap^r, 526-bp BamHI/HindIII PCR fragment of *popD* in pUC18	This study
pJG3	Ap^r, BamHI/HindIII fragment from pJG2 cloned into pJG1	This study
pJG4	SmaI fragment (gentamicin) from pUCGm cloned into BamHI site in pJG3	This study
pJG5	EcoRI/HindIII fragment from pJG4 cloned into SmaI site in pEX100T	This study
pJG6	*popB* cloned downstream of *pG* promotor into pIApG	This study
pJG7	*popD* cloned downstream of *pG* promotor into pIApG	This study
pJG8	*popBD* cloned downstream of *pG* promotor into pIApG	This study
pJG9	Ap^r, 1,615-bp SmaI PCR fragment containing *pcrV* in pEX100T	This study
pJG10	Deletion of codons 17 to 171 of *pcrV* by digestion with SacI-SalI in pJG9	This study
pJG11	*pcrV* cloned downstream of *pG* promotor into pIApG	This study

key component of the TTSS and a potential therapeutic target (21, 45, 50). PcrV is necessary for ExoU-dependent cytotoxicity (45) and translocation of ExoS into epithelial cells (52). However, the exact role played by PcrV in the translocation process is still obscure.

In this work we further characterized the requirement for secreted products encoded by the *pcrGVH-popBD* operon for the pore-forming activity of the *P. aeruginosa* TTSS by constructing specific nonpolar mutants and analyzing them in two cellular infection models. We found that each of three proteins, PcrV, PopB, and PopD, is absolutely required for pore formation and cytotoxicity. Fractionation experiments with infected red blood cells (RBCs) showed that only PopB and PopD form the membrane-associated core of the pore. We demonstrated that PcrV is required for anchoring of PopD into RBC membranes. In addition, immunoprecipitation experiments revealed that there is a direct interaction between PopD and PopB. Consequently, in vivo, PcrV is necessary for functional assembly of a membrane-inserted PopB/PopD complex.

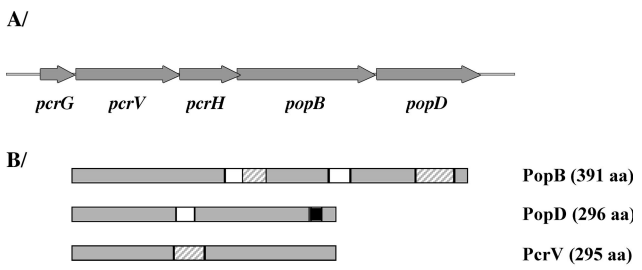

FIG. 1. Genetic organization and structural features of the *P. aeruginosa* type III translocon. (A) Genetic organization of the *pcrGVH-popBD* operon. (B) Predicted transmembrane domains (open boxes), coil-coiled domains (cross-hatched boxes), and amphipathic α-helix (solid box) of secreted proteins from the operon (PopB, PopD, and PcrV). aa, amino acids.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All *P. aeruginosa* strains used in this study (Table 1) were derived from the cytotoxic cystic fibrosis isolate CHA (14, 54), which is referred to below as the wild-type strain. Bacteria were grown either on *Pseudomonas* isolation agar (Difco) plates or in liquid Luria broth (LB) at 37°C with agitation. The antibiotics used for selection were carbenicillin (300 μg/ml) and gentamicin (200 μg/ml). *Escherichia coli* DH5α (Invitrogen) was used for standard cloning experiments.

Construction of CHAΔBD and CHAΔV mutants. All plasmids and primers used in this study are listed in Tables 1 and 2, respectively. The CHAΔBD mutant

TABLE 2. Oligonucleotides used in this study

Primer	DNA sequence[a]
OG1	5′-TCGGATCCGTGATGTTGCGTCGGC
OG2	5′-TCGGATCCGCAGGGTGTCTTCGG
5′*popD*-HindIII	5′-GTCGTAGGGAAGCTTGCAGAA
3′*popD*-BamHI	5′-GAGTCTGCGGGATCCCCGGAGT
5′*popB*-EcoRI	5′-TCCGGAATTCCAGGCACTGTGCA
3′*popB*-BamHI	5′-CCACTCCGGGGATCCCGCAGACT
5′*popB*-XbaI	5′-CTAGTCTAGATAACCGCGAGAAAGGATC
3′*popB*-HindIII	5′-TACCCAAGCTTACGTCTCCTCAGATCGC
5′*popD*-XbaI	5′-GCTAGTCTAGAGATCTGAGGAGACGTCAC
3′*popD*-HindIII	5′-TACCCAAGCTTAGACGGCTCAGACCACT
5′*pcrV*-XbaI	5′-CTAGTCTAGAGTGGCTTGTTGATCTGAGG AATC
3′*pcrV*-HindIII	5′-TACCCAAGCTTCTTTAGATCGCGCTGAGA ATGTCG
5′SmaI-*pcrG*	5′-TGACCCGGGATGGGCGACATGAACGAAT ACAC
3′SmaI-2	5′-TACCCGGGCCGAGTAGAAGC

[a] Restriction sites incorporated into primers are underlined.

was created by using the following strategy. The 5′ flanking region of *popB* and the 3′ flanking region of *popD* were amplified in two separate PCRs by using pIA60 as the template. The oligonucleotides used to amplify the 5′ flanking region of *popB* were 5′*popB*-EcoRI and 3′*popB*-BamHI. PCR amplification generated a unique 426-bp product that was cloned in pUC18 to give pJG1. Amplification of the 3′ flanking region of *popD* was achieved with 5′*popD*-HindIII and 3′*popD*-BamHI, which generated a 526-bp fragment that was cloned in pUC18, generating pJG2. The two PCR products were ligated together in pUC18, giving plasmid pJG3. Next, an 855-bp SmaI fragment containing the gentamicin resistance cassette from pUCGm was ligated into the blunt-ended BamHI site of pJG3, giving pJG4. To carry out gene replacement on the *P. aeruginosa* chromosome, the EcoRI-HindIII insert from pJG4 was blunt ended with the Klenow enzyme and subcloned into pEX100T. The resulting suicide plasmid, pJG5, was then transferred to *P. aeruginosa* CHA by triparental mating by using pRK2013 as a helper plasmid, as described previously (34). Double recombinants were isolated by a negative selection strategy by using *Pseudomonas* isolation agar plates containing 5% sucrose as described previously (49). The correct double recombination event at the *popBD* locus was verified by Southern blotting.

The *pcrV* gene in the parental CHA strain was inactivated by using the following strategy. A PCR-amplified fragment containing the *pcrV* gene with 308 bp of upstream DNA and 410 bp of downstream DNA was generated by using primers 5′SmaI-*pcrG* and 3′SmaI-2. The amplified 1,615-bp PCR fragment was cloned as an SmaI-SmaI insert into pEX100T, yielding pJG9. A deletion of codons 17 to 171 was then generated by digestion of pJG9 with SacI-SalI and blunt ending with the Klenow enzyme, and this was followed by religation. The resulting suicide plasmid, pJG10, was then transferred to *P. aeruginosa* CHA, as described above. Clones with pJG10 integrated into *pcrV* by a single recombination event were selected by using *Pseudomonas* isolation agar plates containing carbenicillin. Selection for a second recombination event was performed by growing cointegrate strains on plates containing 5% sucrose. Carbenicillin-sensitive, sucrose-resistant strains were tested for correct replacement of the wild-type allele by the *pcrV*-deleted allele by Western and Southern blotting. Several independently constructed mutants were tested in preliminary experiments and had the same phenotype.

Construction of complementing plasmids. pIApG was constructed by placing the PCR-amplified promoter region of *pcrGVH-popBD* upstream of *gfp*mut3 in pIA101*, as described previously (15), by using the OG1 and OG2 oligonucleotides (Table 2). All genes were amplified from pIA60 by using Vent polymerase (BioLabs) and oligonucleotides listed in Table 2. Unique restriction sites were introduced and used to clone each amplified fragment into the appropriate vector's multiple cloning sites. All complementing plasmids were constructed in pIApG after removal of the *gfp* gene, which placed the gene of interest under transcriptional control of a native promoter of the *pcrGVH-popBD* operon (*pG*). All cloning was performed in *E. coli* DH5α. Complementing plasmids were introduced into *P. aeruginosa* strains by transformation (10).

Production and purification of antibodies. Recombinant proteins PcrV, PopB, and PopD containing His_6 tags were overproduced in *E. coli* BL21(DE3) (Invitrogen). Details of plasmid construction, the induction conditions, and the purification procedures have been previously described (47). Antibodies were raised in rabbits for purified His_6-PcrV and gel-excised His_6-PopB and His_6-PopD antigens by Eurogentec as described by the manufacturer. Specific anti-PcrV and anti-PopB antibodies were affinity purified from the serum by using a preactivated CH Sepharose 4B gel (Amersham Pharmacia) coupled with recombinant purified His_6-PcrV or His_6-PopB, as described in the manufacturer's protocol.

Immunoblot analysis. Overnight cultures of *Pseudomonas* strains grown at 37°C in LB supplemented with appropriate antibiotics were diluted to an optical density at 600 nm (OD_{600}) of 0.2 in LB or calcium-depleted LB (induction conditions) containing 5 mM EGTA and 20 mM $MgCl_2$ and then grown at 37°C until the OD_{600} reached 0.8. After the OD_{600} was measured, the cells were harvested, and the culture supernatant was collected. The proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to nitrocellulose membranes with 1× Laemmli buffer containing 20% ethanol. The membranes were blocked with 5% nonfat dry milk before addition of polyclonal primary antibodies and a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma). Detection was performed with an ECL kit (Amersham Pharmacia). The detection limit with each antibody was evaluated by using purified recombinant PcrV, PopB, and PopD. The antibodies used were affinity-purified anti-PcrV (1:3,000) and anti-PopB (1:5,000) and total polyclonal sera raised against PopD (1:1,000) and ExoS (1:1,000) (kindly provided by A. Forsberg [22]).

Cell culture and infections. NIH 3T3 fibroblasts expressing enhanced green fluorescent protein (GFP)–β-actin (3T3/GFA) were a gift from R. Scaife (46). The cells were cultured in Dulbecco modified Eagle medium (Gibco) supplemented with heat-inactivated 10% fetal calf serum (Gibco). The cells were seeded in four-well Lab-Tek I chambers (Nunc) 24 h before infection. Bacterial strains were grown overnight in LB, diluted to an OD_{600} of 0.1, and grown for an additional 3 h to an OD_{600} of approximately 1.2. 3T3/GFA cells were infected with bacteria in Dulbecco modified Eagle medium at a multiplicity of infection (MOI) of 10. Modifications in cell morphology and GFP-actin distribution were monitored by using a Leica inverted microscope (DM IRE 2). The images were collected by using a DC 350F digital camera and were treated with QFluo Pro software.

Hemolysis assay. Sheep RBCs, obtained from BioMérieux, were washed three times in phosphate-buffered saline (PBS; 150 mM NaCl; pH 7.4) and resuspended in RPMI 1640 medium (Sigma) at a concentration of 5×10^8 RBCs ml^{-1} at 4°C. Bacteria were grown in LB to an OD_{600} of 1.2, centrifuged, and resuspended in RPMI 1640 medium at a concentration of 5×10^8 bacteria ml^{-1}. Hemolysis assays were initiated by mixing 100 μl of RBCs and 100 μl of bacteria in round-bottom 96-well plates, which were then centrifuged at $2,000 \times g$ for 10 min and incubated at 37°C for 1 h. The release of hemoglobin was measured, and the percentage of hemolysis was calculated as described previously (4).

RBC membrane isolation. RBC membrane isolation was performed essentially as described previously (4), with some modifications. Bacteria and sheep RBCs were resuspended in Tris-saline (30 mM Tris, 150 mM NaCl; pH 7.5) at concentrations of 5×10^{10} and 1×10^{10} cells ml^{-1}, respectively. Hemolytic reaction mixtures were prepared in 50-ml conical tubes with 2×10^9 (each) bacteria and RBCs and a protease inhibitor cocktail (Complete; Roche). Samples were centrifuged at $2,000 \times g$ at 4°C for 10 min and incubated at 37°C for 1 h. Hemolysis was assessed spectrophotometrically as described above, after resuspension and centrifugation. Two milliliters of distilled water was added to each sample to lyse all RBCs, and the preparations were vortexed and centrifuged again to remove the bacteria. A total of 3.5 ml of supernatant was collected and deposited on top of a discontinuous sucrose gradient consisting of 4 ml of 44% sucrose and 4 ml of 25% sucrose in Tris-saline containing the protease inhibitor cocktail in an SW41 centrifuge tube (Beckman). The gradients were centrifuged at $15,000 \times g$ for 16 h at 4°C. The material at the 44% sucrose–25% sucrose interface was collected, diluted in Tris-saline, and concentrated by centrifugation at $450,000 \times g$ for 20 min at 4°C in a TLA 100.3 rotor (Beckman). The pellets were resuspended in 100 μl of Laemmli reducing sample buffer. The protein contents were separated by SDS-PAGE and visualized by Western blotting. To assess the strength of association of Pop proteins with RBC membranes, 100 μl of lysed RBC membranes, isolated after infection, was incubated at 4°C for 1 h in Tris-saline or Tris-saline containing 5 M NaCl or 0.2 M Na_2CO_3 (pH 11.0). After incubation, the material was diluted in Tris-saline and concentrated by centrifugation for 20 min at $450,000 \times g$ at 4°C. The pellets were resuspended in a minimal volume of loading buffer, and the protein contents were separated by SDS-PAGE and visualized by Western blotting. In these experiments, the CHAΔBD/*popBD* strain was used since more Pops could be detected in RBC membranes after infection.

Immunoprecipitation. Liposomes containing 5% cholesterol, 35% phosphatidylcholine, and 60% phosphatidylserine (Sigma) were prepared by drying the phospholipids under N_2 and resuspending them by sonication in PBS at a concentration of 2 mg/ml. Recombinant PcrV, PopB, and PopD separated from their corresponding chaperones were obtained as described previously (47), with one modification: the His tag was removed from PcrV by thrombin digestion before ion-exchange chromatography. For immunoprecipitation, liposomes (final concentration, 1 mg/ml) were incubated with PcrV and/or PopB and/or PopD (final concentration of each, 30 μg/ml) in 100 μl of PBS at room temperature for 2 h. Liposomes were collected by centrifugation at $150,000 \times g$ and 4°C for 20 min. After removal of the supernatants containing proteins not bound to the liposomes, the proteoliposome pellets were solubilized in 1 ml of PBS containing 500 mM NaCl and 1% Triton X-100 (PBS-N-T). Three hundred microliters of this extract was incubated for 2 h at 4°C with 4 μl of EZview Red protein A affinity gel (Sigma) and 1 μg of affinity-purified PopD antibodies. The protein A beads were collected by centrifugation and washed twice with PBS-N-T and once with PBS. Finally, the beads were resuspended in 50 μl of Laemmli reducing loading buffer, and 15 μl was separated by SDS-PAGE. PcrV, PopB, and PopD were detected by Western blotting by using the procedures described above. The amounts of PcrV, PopB, and PopD in the positive control lane were 5, 5, and 10 ng, respectively.

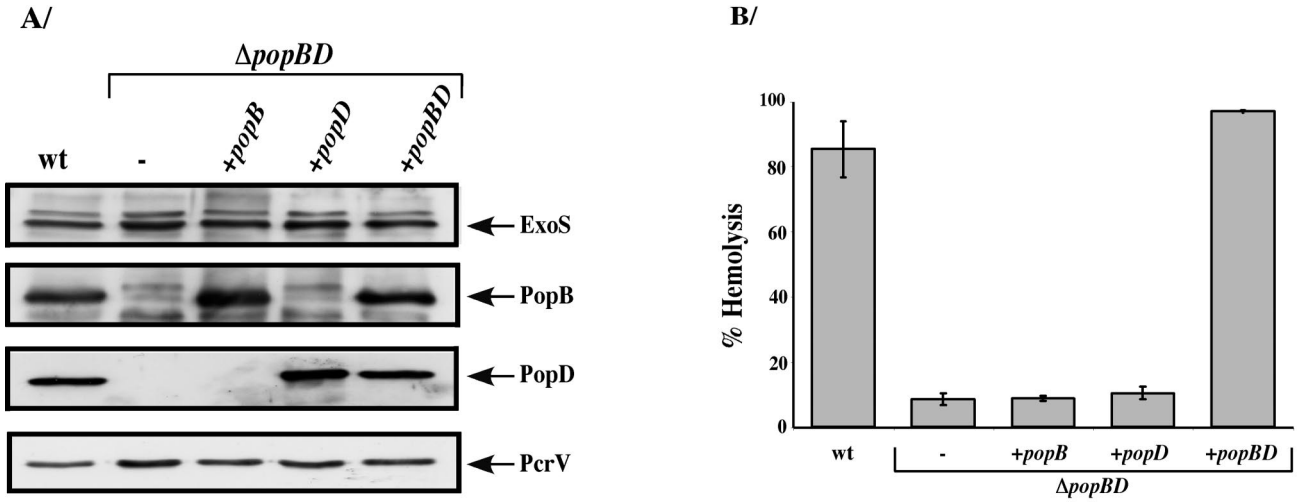

FIG. 2. Characterization of the CHAΔBD mutant and complemented strains. (A) Western blot analysis of secreted proteins from *P. aeruginosa* strains cultivated under TTSS-inducing conditions (in LB supplemented with 5 mM EGTA and 20 mM $MgCl_2$). Portions (25 μl) of supernatants were directly resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and developed by Western blotting with affinity-purified anti-PcrV antibodies and total polyclonal serum raised against ExoS, PopD, and PopB. (B) Hemolytic activities of *P. aeruginosa* strains. Sheep RBCs were infected at an MOI of 1 and incubated at 37°C for 1 h. Lysis was assessed by measuring hemoglobin release into infection supernatants spectrophotometrically at 540 nm. The values are the means of at least three independent experiments, and the error bars indicate standard deviations. wt, wild type.

RESULTS

Simultaneous requirement for PopB and PopD in pore formation. In a previous report it was shown that a *P. aeruginosa* CHA mutant having a transposon insertion in the *pcrV* gene did not exhibit pore-forming activity on macrophages and lytic activity on erythrocytes. This mutant, however, exhibited a polar phenotype with respect to downstream genes, and consequently, neither PopB nor PopD was found to be secreted (16). To address the role of each individual protein in pore formation, we first constructed a double PopB/PopD mutation in the cytotoxic CHA strain and then complemented this mutant in *trans* with either *popB* or *popD* or with both genes (see Materials and Methods). For complementation studies, all genes were amplified by PCR and cloned downstream of the native promoter of the operon, *ppcrG*, into a pUCP20-based vector (Tables 1 and 2). The level of secretion of proteins in vitro was assessed by Western blotting by using specific polyclonal antibodies raised in rabbits against recombinant PcrV, PopB, PopD, and ExoS proteins (see Materials and Methods). Bacterial cultures were grown in LB containing EGTA, a Ca^{2+}-chelating agent known to induce secretion of TTSS proteins (56, 60). As shown in Fig. 2A, CHAΔBD did not secrete PopB and PopD but secreted wild-type quantities of PcrV, showing that the mutation specifically affected Pop proteins and that the secretion apparatus was intact. Indeed, all isogenic strains secreted the same quantities of the effector ExoS. When provided in *trans* either individually or together in the CHAΔBD strain, both PopB and PopD were secreted. Neither mutation influenced Ca^{2+}-dependent regulation of secretion (data not shown). We then tested the mutant and the complemented strains for the ability to provoke lysis of RBCs. Measurement of pore formation by contact-dependent TTSS by using RBCs has been reported to be an efficient and simple method that is frequently used when the translocation machinery is studied (58). The parental strain CHA lysed 80 to 90% of the RBCs within 1 h when it was added at an MOI of 1. As predicted, only a basal level of hemoglobin release, which was a consequence of a type III-independent hemolytic activity (16), was detected from CHAΔBD-infected cells. Full restoration of wild-type hemolysis was achieved only when both genes, *popB* and *popD*, were provided in *trans* (Fig. 2B).

The translocation of Exo effectors into eukaryotic cells was tested in parallel for all strains. Delivery of ExoS and ExoT into host cells has a profound effect on the cellular shape and on the actin network due to the GTPase-activating activity toward small GTP-binding proteins of the Rho family (25, 42, 52). We took advantage of NIH 3T3 fibroblasts which constitutively express GFP fused to actin (3T3/GFA) in order to monitor in vivo cell modifications during infection by fluorescent microscopy. Within 2 h postinfection with the parental CHA strain, 3T3/GFA cells showed visible perturbations of actin fibers, resulting in formation of highly fluorescent GFP-actin patches (Fig. 3). The double mutant secreting only PcrV was unable to disrupt the actin cytoskeleton of 3T3/GFA cells. Only complementation with both the *popB* and *popD* genes restored the wild-type cytotoxic activity. Strains secreting combinations of PcrV and PopB or PcrV and PopD were unable to destabilize RBC membranes or to provoke any actin rearrangement (Fig. 2B and 3). Mixing the strain secreting a combination of PcrV and PopD and the strain secreting a combination of PcrV and PopB at a 1:1 ratio prior to infection did not result in restoration of lytic activity or translocation (data not shown).

PcrV is required for pore formation but not for PopB and PopD secretion. To study the function of PcrV in translocation pore formation, we constructed a nonpolar and nonmarked mutant in which the *pcrV* gene in the chromosome of *P. aeruginosa* CHA was inactivated by deleting an internal part of the

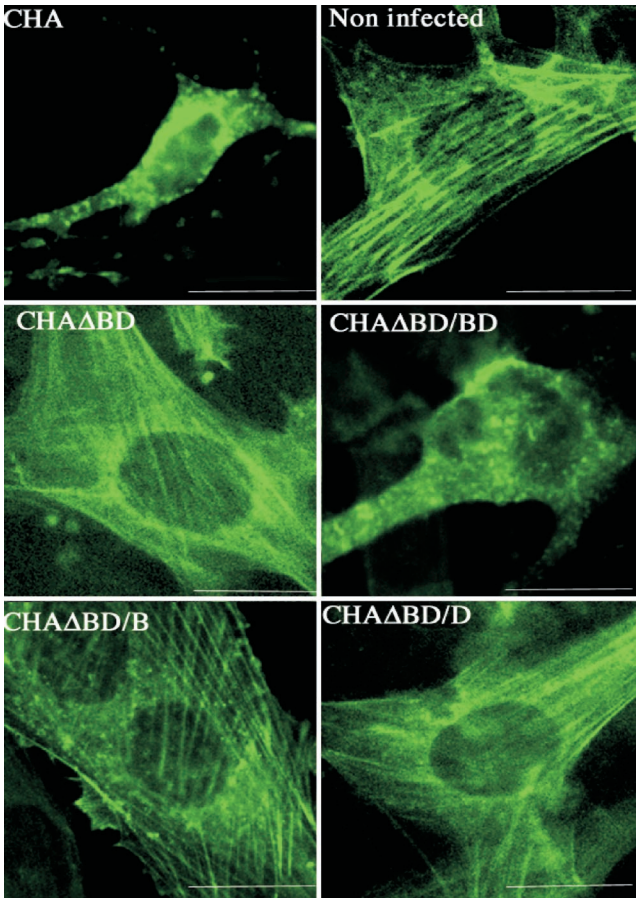

FIG. 3. Translocation of Exo effectors into NIH 3T3 fibroblasts. NIH 3T3 cells expressing constitutively an enhanced GFP–β-actin fusion were seeded into Lab-Tek I chambers 24 h before infection. Cells were infected with the indicated strains at an MOI of 10. Modifications in cell morphology and GFP-actin distribution were monitored by using a Leica inverted microscope (DM IRE 2). The images were collected by using a DC 350F digital camera and were treated with the QFluo Pro software. Scale bars = 20 μm.

gene (see Materials and Methods). Western blotting with affinity-purified anti-PcrV antibodies was used to screen for double recombinants that did not synthesize PcrV in vitro. To compare the secretion profiles of the parental strain CHA and the CHAΔV mutant, supernatants from bacterial cultures, grown in LB under inducing or noninducing conditions for TTSS secretion, were directly analyzed by Western blotting. The absence of PcrV had no effect on Ca^{2+}-dependent regulation of secretion, since the effector ExoS and Pop proteins were secreted only when the bacteria were grown in medium containing the Ca^{2+}-chelating agent EGTA (Fig. 4A). Similar quantities of ExoS, PopB, and PopD were found in the supernatants of all strains. These results demonstrate that the PcrV protein is not implicated in the regulation of TTSS expression or in the secretion of the PopB/D proteins. When tested in infection models with RBCs and 3T3/GFA cells (Fig. 4B and C), the mutant did not display any pore-forming activity, confirming two previous reports in which PcrV was found to be necessary for ExoU- and ExoS-dependent cytotoxicity of cultured cells (45, 52). Complementation of the mutant with the wild-type *pcrV* gene restored hemolysis and cytotoxicity to the parental strain levels (Fig. 4B and C). In summary, PopB and PopD secreted in the absence of PcrV were unable to induce pore formation. No restoration of lytic activity or translocation could be obtained by coinfection with the CHAΔBD and CHAΔV strains (data not shown). Together with data for the CHAΔBD mutant, these results indicate that PcrV, PopB, and PopD contribute simultaneously to the assembly of a pore capable of performing hemolysis and effector trafficking.

PcrV is required for functional assembly of the Pop pore in RBC membranes. Since the CHAΔV mutant was not able to provoke any membrane destabilization or effector translocation but secreted translocators PopB and PopD in vitro, we hypothesized that the PcrV protein could help insertion and/or stabilization of the Pop pore within host membranes. To test this hypothesis, RBC membrane fractions were examined for the presence of PcrV, PopB, and PopD after infection with CHA (wild type), CHAΔBD, and CHAΔV. Hemolysis was systematically checked at 1 h postinfection. The membrane fractions were recovered from infected RBCs by using a discontinuous sucrose gradient and were analyzed by Western blotting for the presence of Pops and PcrV (Fig. 5A). PopB and PopD were readily detected in RBC membranes infected by parental strain CHA. In order to study the type of association of Pop proteins with lipid bilayers, the isolated membranes were incubated with agents known to detach peripheral membrane proteins. After incubation with 5 M NaCl or 0.2 M carbonate (pH 11), the majority of the PopB and PopD proteins remained associated with the membranes (Fig. 5, compare lane ΔBD/*popBD* before treatment and lanes NaCl and pH 11 after treatment). This strong association and the presence of two and one predicted transmembrane domains in PopB and PopD, respectively, indicate that these proteins are inserted in RBC membranes.

Unlike Pops, PcrV was never found in association with RBC membranes (Fig. 5A). To be sure that the absence of PcrV from membranes was not an artifact due to less efficient detection of the protein by antibodies, the detection limits of anti-PcrV and anti-PopB antibodies in Western blots were compared by using purified recombinant proteins. At a given dilution the anti-PcrV and anti-PopB antibodies had comparable detection limits (data not shown).

Notably, in membranes infected by the PcrV-deficient mutant, PopB was found to be membrane associated, indicating that PcrV is not necessary for insertion of PopB into lipid bilayers. On the other hand, PopD was absent from the CHAΔV-infected RBC membranes, suggesting that stable association of PopD with cellular lipid bilayers requires a functional PcrV. Indeed, the localization of PopD within membranes was restored in a complemented ΔV strain. The absence of only PopD from infecting bacteria (CHAΔBD/B) did not affect the association of PopB with RBC membranes. The same was found for PopB, whose absence did not influence the ability of PopD to get inserted into membranes (Fig. 5A). From these experiments, we concluded that the membrane part of the translocon is composed of both PopB and PopD and that PcrV is required for the insertion and/or stability of PopD within host membranes.

PopD directly interacts with PopB within liposomes. Immunoprecipitation experiments were performed in order to study

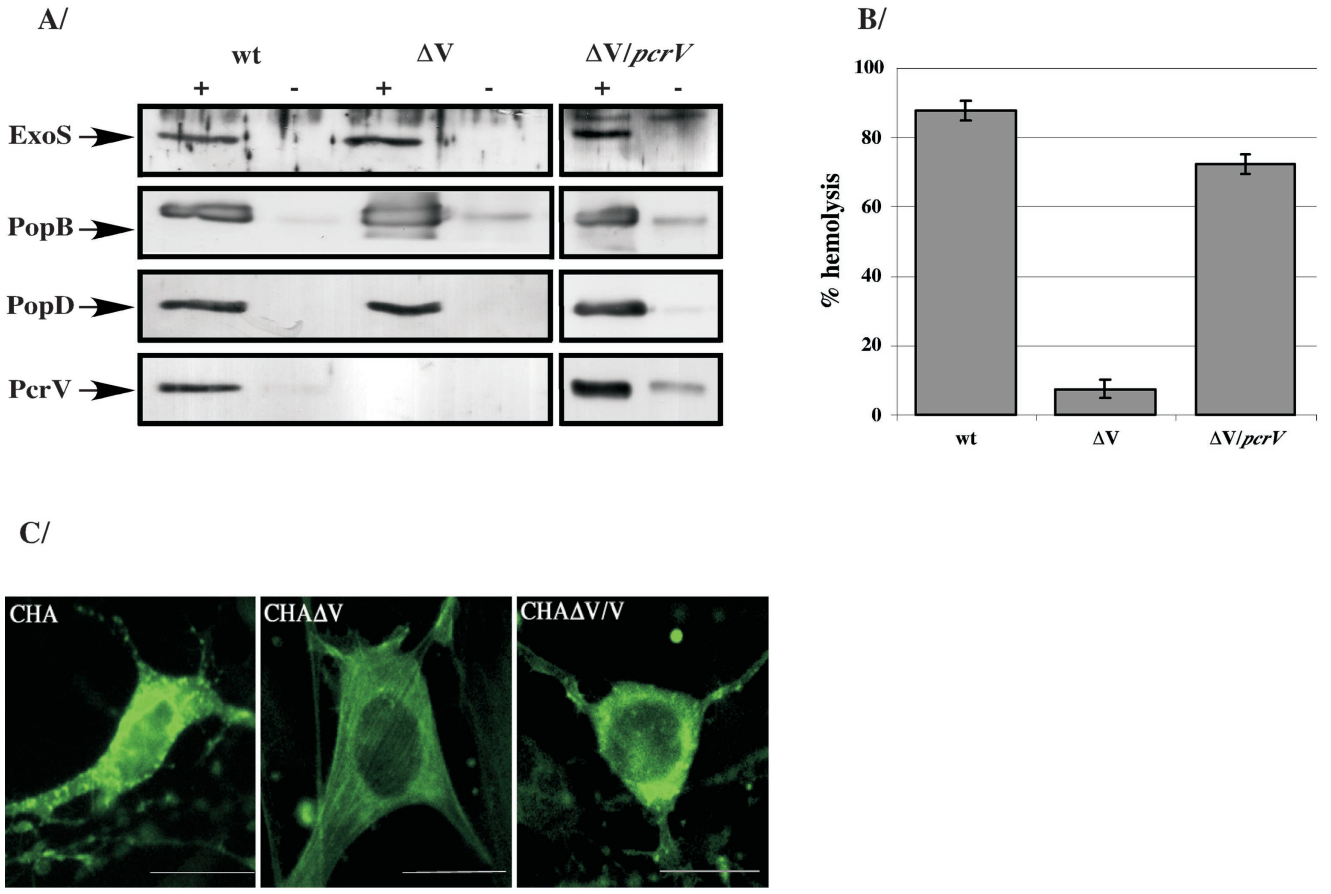

FIG. 4. CHAΔV secrets PopB and PopD but is incapable of pore formation. (A) Secretion of ExoS, PopB, PopD, and PcrV by CHA (wild type), CHAΔV, and complemented strain CHAΔV/*pcrV*. Western blot analysis was performed with culture supernatants of the strains indicated grown in noninducing conditions (LB) (lanes −) and in LB supplemented with EGTA (lanes +), representing TTSS-inducing conditions. (B) RBCs were infected with the different *P. aeruginosa* strains indicated at an MOI of 1. Hemolysis was measured after 1 h of incubation at 37°C. The values are means of three independent experiments, and the error bars indicate standard deviations. (C) 3T3/GFA cells were infected at an MOI of 10 and observed for modifications of the actin cytoskeleton 2 h postinfection, as described in the legend to Fig. 3. Scale bars = 20 μm. wt, wild type.

the interactions among PcrV, PopB, and PopD (Fig. 6). As too few proteins were retained in the infected RBC membrane fraction, we used an in vitro liposome model of PopB/D pore assembly. We have shown previously that PopB and PopD can be purified in vitro in a complex with their cognate chaperone, PcrH. In acidic conditions, PopB and PopD dissociate from the chaperone and oligomerize. Furthermore, when they are in the presence of liposomes, PopB and PopD are able to form ring-like structures (47). Here, liposomes were incubated with different combinations of PcrV and/or PopB and PopD prepared in the oligomeric state. Subsequently, proteoliposomes were pelleted to purify the bound fraction and were solubilized with Triton X-100 to eliminate the possible cointeractions via lipid molecules. The corresponding protein extracts were subjected to immunoprecipitation. Antibodies raised against PopD were able to coprecipitate PopD and significant amounts of PopB from liposomes previously incubated with PcrV, PopB, and PopD (Fig. 6, lane 1). The specificity of this coprecipitation was examined by performing the same procedure with extracts lacking PopD. Anti-PopD antibodies failed to precipitate PopB from these extracts, showing that no cross-reaction between anti-PopD antibodies and PopB occurs (Fig. 6, lane 2). Notably, coprecipitation of PopD and PopB was detected in the absence of PcrV (Fig. 6, lane 3), indicating that the PopB-PopD interaction is PcrV independent. Moreover, PcrV did not coprecipitate with PopD in the presence or absence of PopB (Fig. 6, lanes 1 and 4). These results provide strong evidence that PopD directly interacts with PopB but not with PcrV.

DISCUSSION

Type III secretion is a mechanism widely used by gram-negative pathogens to intoxicate eukaryotic cells and permits successful multiplication of the pathogen in the host. In *P. aeruginosa*, components encoded in the TTSS locus are required for efficient killing of phagocytes, lysis of RBCs, and intoxication of epithelial and endothelial cells. Different mechanisms leading to either oncosis or apoptosis of host cells have been described, and all of these mechanisms depend on proteins secreted by type III machinery.

In this work we shed light on the role of essential secreted proteins encoded within the *pcrGVH-popBD* translocation operon in *P. aeruginosa* TTSS-dependent cytotoxicity. We em-

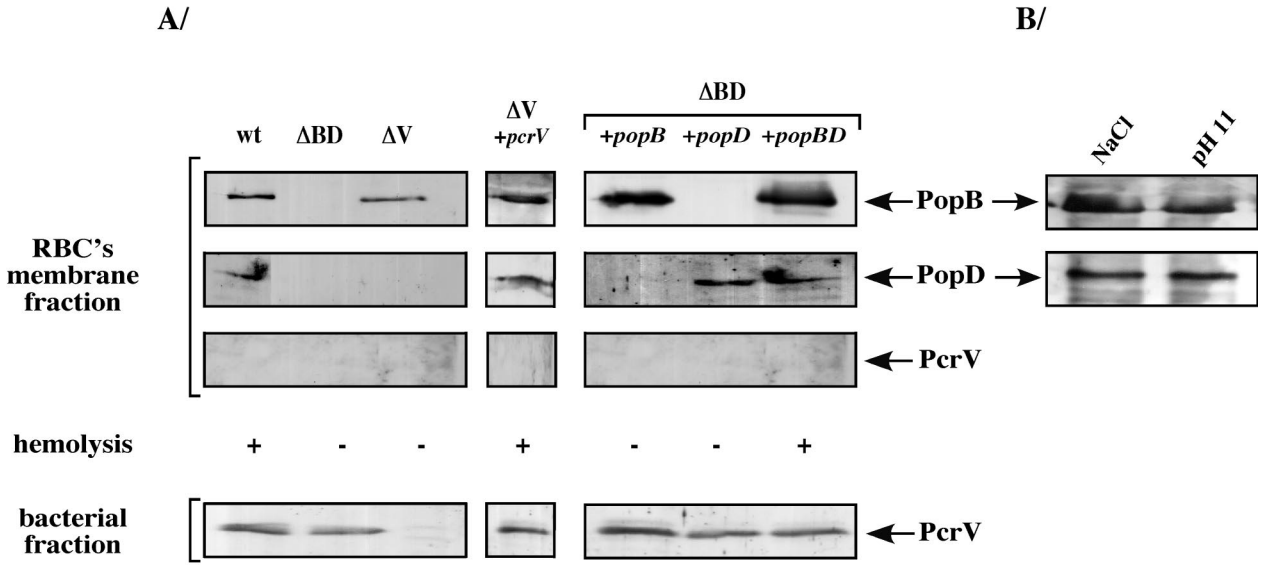

FIG. 5. Presence of PopB, PopD, and PcrV in RBC membrane fractions. (A) RBCs were infected with the different *P. aeruginosa* strains indicated at an MOI of 1 for 1 h at 37°C. Hemolysis was assayed for each reaction before total lysis was achieved by addition of sterile water. Membranes were recovered after centrifugation at the 44% sucrose–25% sucrose interface and were concentrated by ultracentrifugation. (B) Association of Pop proteins with RBC membranes isolated after infection and stripped with 5 M NaCl (lane NaCl) and 0.2 M carbonate, pH 11.0 (lane pH 11). Membrane-associated proteins were separated by SDS-PAGE and subjected to Western blot analysis by using affinity-purified PcrV and PopB antibodies and total polyclonal serum raised against PopD. The hemolysis values were >70% for lanes + and <20% for lanes −. wt, wild type.

ployed two cellular infection models to analyze specific mutations constructed within the operon. Fibroblasts constitutively expressing actin fused to GFP proved to be a good tool to monitor translocation of effectors, since perturbation of the actin fibers due to ExoS and/or ExoT effectors could be easily visualized by fluorescence microscopy in vivo. Measurement of hemolysis with sheep RBCs was used to evaluate pore formation, and the results displayed a good correlation with effector translocation. We found that there was a simultaneous requirement for all three proteins, PcrV, PopB, and PopD, for cell intoxication and hemolysis. Individually secreted pairs of proteins (PcrV and PopB, PcrV and PopD, or PopB and PopD) were not able to destabilize RBC membranes and did not support efficient translocation of Exo effectors. Thus, each of the three secreted proteins encoded by the *pcrGVH-popBD* operon is essential for full *P. aeruginosa* cytotoxicity. In recently published work, it was shown that in vitro, PopB and PopD were able to form ringlike structures and lyse liposomal membranes individually (47). The requirement for the simultaneous presence of all three proteins in vivo may reflect a difference in membrane composition or may be directly related to the fact that very large amounts of soluble Pop proteins were employed for in vitro experiments, exacerbating their activity toward membranes.

The secreted proteins PcrV, PopB, and PopD exhibit high degrees of sequence similarity with three well-studied proteins of *Yersinia* species, LcrV, YopB, and YopD, respectively (23). Several groups have proposed that LcrV, YopB, and YopD participate in formation of a channel by which the toxins cross the host plasma membrane (39, 41, 53). However, the *Yersinia* translocation pore has never been visualized, and the molecular composition of the pore is controversial. YopB and YopD expressed by *Yersinia* spp. were found to be associated with liposomes after infection (53), and YopB was responsible for lysis of RBCs (26).

Examination of the wild-type *P. aeruginosa*-infected RBC membranes indicated that the membrane core components of the translocation pore are most probably PopB and PopD since both proteins are found in membrane fractions. Moreover, incubation of PopB/PopD-containing membranes with a high salt concentration (5 M NaCl) and at an alkaline pH (0.2 M Na_2CO_3, pH 11), which are known to dissociate proteins that are peripherally associated with membranes via electro-

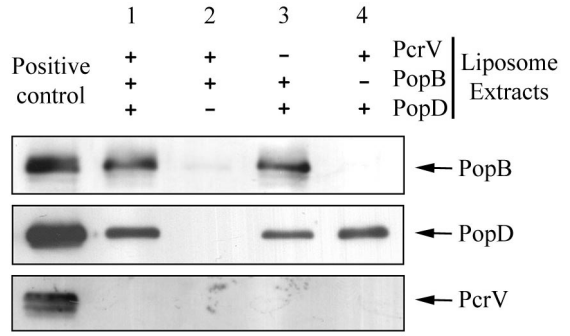

FIG. 6. Coimmunoprecipitation of PopD and PopB. Liposomes were incubated with different combinations of recombinant PcrV, PopB, and PopD and then solubilized with Triton X-100. The corresponding extracts containing all three proteins (lane 1) or lacking PopD (lane 2), PcrV (lane 3), or PopB (lane 4) were incubated with affinity-purified antibodies against PopD and beads of protein A. Precipitated proteins were analyzed by SDS-PAGE, followed by immunoblotting with antibodies against PcrV, PopB, and PopD. Purified recombinant proteins were used to monitor antibody reactivity (positive controls).

static and hydrophilic interactions, respectively (33, 51), did not disrupt the association of Pops with RBC membranes. Importantly, we demonstrate here that in ex vivo infection models, although each protein individually was able to associate with RBC membranes in the absence of another protein, no functional pore could be detected by a hemolysis assay unless both proteins were exported by the same bacterium. This could be due to the necessity for PopB-PopD interactions to form a functional pore. To test this hypothesis, we took advantage of the fact that recombinant PopB and PopD proteins oligomerize and incorporate within liposomes in vitro (47) to examine the possible interactions between PopB and PopD under conditions close to the conditions for in vivo assembly of the translocon. Indeed, not enough proteins could be recovered from the RBC membranes to determine such interactions. Significantly, our coimmunoprecipitation experiments clearly showed that PopD directly interacts with PopB. Thus, the functional translocation pore must be composed of PopB and PopD assembled into a macromolecular complex.

Construction and characterization of the nonpolar, nonmarked deletion mutant CHAΔV allowed us to obtain new insights into the function of PcrV in pore formation. The V antigen is unique and is the key component in the TTSS of *Yersinia* spp. and *P. aeruginosa*. The fact that antibodies directed towards PcrV (21, 45) and LcrV (31, 32) protect model animals against infection suggests that this protein is a possible target for alternative antibacterial treatment. Indeed, LcrV has been considered for several decades to be a major antigen of *Yersinia pestis*, and its use in vaccine-related prophylaxis against plague is being actively investigated (2, 31). Although numerous studies have contributed to our understanding of the role of *Yersinia* V protein in the regulation of Yop secretion and in Yop effector translocation, no precise and clear function has been attributed to this protein. In this work, we took advantage of the fact that deletion of *pcrV* has no effect on the expression of genes encoding PopB and PopD to investigate the role of PcrV in pore formation. In the PcrV-deficient mutant, PopB and PopD, as well as the ExoS toxin, were found to be secreted only in the absence of Ca^{2+}. In disagreement with two recent reports (38, 45), the regulatory features of the PcrV mutant were indistinguishable from those of the parental strain. The same phenotype was found in reference strain PAO1 in which *pcrV* was deleted in the same manner (data not shown) and in a *pcrV* mutant of strain PAK (52). It is conceivable that the role of PcrV in the expression of TTSS genes is either culture condition dependent or strain dependent. Indeed, McCaw et al. (38) and Sawa et al. (45) used the same mutant that is a derivative of PA103 (an ExoU- and ExoT-positive strain), while the CHA, PAO1, and PAK strains are ExoS and ExoT positive.

Although CHAΔV secreted Pop proteins, it was not cytotoxic toward fibroblasts and was unable to provoke hemolysis. Thus, in CHAΔV the whole secretion apparatus was assembled and functional, but the translocation step was impaired. Furthermore, infected RBC fractionation experiments demonstrated that only PopB and PopD were associated with the host cell membranes. Although easily detected in bacterial culture supernatants with affinity-purified polyclonal antibodies, PcrV was never detected in membrane fractions even when the membrane sample was overloaded on an SDS-PAGE gel, in agreement with the fact that in our hands purified PcrV was not able to associate with artificial liposomes (47) and the fact that this protein is accessible to protective antibodies added in vitro (45). However, the work described here suggests that PcrV, although not present within host cell membranes, is needed for assembly of the functional pore in vivo by facilitating stable insertion of PopD within membranes, since the PcrV mutant inserted only PopB into RBC membranes.

We suggest that PcrV may act somewhere between the type III needle and the PopB/D pore complex. However, this protein is not needed for a direct interaction between the bacterial secretion complex and host membranes, since PopB is found within RBC membranes even in the absence of PcrV.

No coprecipitation of PcrV with PopD could be detected, confirming two previous reports in which no interaction between Pop proteins and PcrV was found by using either affinity purification procedures (47) or immuno-overlay assays (1). It is conceivable that an unidentified protein(s) could act as an intermediate between PcrV and Pop proteins. However, the possibility that the interactions between these proteins are too labile to be detected by the methods employed so far cannot be excluded. Considering the similarities between the translocation systems of *Yersinia* and *P. aeruginosa* (6, 8), it would be interesting to determine whether LcrV participates in assembly of the functional YopB/D translocon in *Yersinia* spp. in the same manner. Although LcrV was originally implicated in the regulation and secretion of translocators, recent work of Marenne et al. showed that in an engineered poly-Yop mutant (the Null strain) the LcrV deficiency did not have an effect on YopB and YopD secretion but abolished pore formation (37). Moreover, although recently contradicted (35), interactions between LcrV and YopB and LcrV and YopD have been demonstrated (44).

In most TTSS of other gram-negative bacteria the translocation process requires three secreted proteins, two of which have been found to be associated with eukaryotic membranes. Examples are EspA, EspB, and EspC of pathogenic *E. coli* (27); SseB, SseC, and SseD encoded by a second TTSS locus of *Salmonella* spp. (40); and IpaB, IpaC, and IpaD of *Shigella flexneri* (4). Although sequence similarities between PcrV/LcrV and components of other TTSS are not evident, it is possible that these molecules play roles similar to those of EspA, SscB, and IpaD, which also lack theoretical hydrophobic domains and are not found associated with membranes. Indeed, in the TTSS of *E. coli*, the interactions between the needle and translocator components involve EspA (17, 27, 59). In conclusion, our work provides new insight into the role of the V antigen, a key TTSS component, in translocation of bacterial effectors across the plasma membrane to the host cell cytoplasm. Biochemical and structural analyses, as well as further experiments to decipher interactions among PcrV, Pops, and the needle component(s), are under way.

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